

AD _____

GRANT NUMBER DAMD17-94-J-4004

TITLE: World Reference Center for Arboviruses

PRINCIPAL INVESTIGATOR: Gregory H. Tignor, Sc.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520-8047

REPORT DATE: July 1997

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSURED 3

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
July 1997

3. REPORT TYPE AND DATES COVERED
Final (9 Dec 93 - 8 Jun 97)

4. TITLE AND SUBTITLE

World Reference Center for Arboviruses

5. FUNDING NUMBERS

DAMD17-94-J-4004

6. AUTHOR(S)

Tignor, Gregory H. Sc.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, Connecticut 06520-8047

8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19980722 070

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. ABSTRACT (Maximum 200 words)

The following objectives were completed: (a) identified and characterized suspected arboviruses from the U.S. and abroad, (b) developed new techniques for virus identification, (c) prepared and distributed reference reagents and PCR protocols and primers, (d) carried out local and international virus surveys. Specific subprojects included (a) nucleotide sequence analysis of flavivirus and arenavirus RNA; (b) developing viral inactivating agents to render clinical samples non-hazardous; (c) use of the reagent bank to identify emerging viruses by conventional and molecular methods; (d) completing an inventory of the entire collection; (e) making an electronic catalogue of the collection; (f) making plans for moving the collection to the CDC.

14. SUBJECT TERMS
Arboviruses, ID, BL3

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT
Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

hbs In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

hbs For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

Where In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Where In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

hbs In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Gregory R. J. J. 1/7/98
PI - Signature Date

TABLE OF CONTENTS

FOREWORD	3
TABLE OF CONTENTS.....	4
A. INTRODUCTION.....	5
B. BODY.....	5
1. Reagents shipped	5
Table 1. Reagents Distributed 1995-1997.....	6
2. DNA primers	7
3. Virus identification	7
5. Completing The Yale Catalogue.....	8
a. General description:	8
Table 2. Data entries in the Yale catalogue.....	8
b. Physical location of the collection.....	9
Table 3. Detailed inventory of storage freezers (-20°C).....	9
6. Local Field Surveillance , 1995-1997	10
a. Rhode Island Arbovirus Surveillance Program: Summary.....	10
Table 4. Summary of mosquito surveillance program.....	10
Table 5. Results of Rhode Island State mosquito-virus isolation studies.....	10
b. Connecticut Arbovirus Surveillance Program: Summary	10
Table 6. Summary of CT State mosquito surveillance program	10
c. Summary Of New England Surveillance Program.....	11
7. International Field Surveillance for Arboviruses.....	11
a. Turkey	11
b. Sri Lanka	12
c. Venezuela	12
d. Evaluation Of A Dengue Fever Intervention In Barbados, W.I.....	13
8. Development of Rapid Diagnostic Reagents.....	13
a. Hemorrhagic Fever PCR.....	13
b. Effects Of Different Virus Inactivating Methods On Arbovirus Antigens.....	14
9. Advances in Molecular Epidemiology.....	14
a. Arenavirus phylogeny	14
b. Dengue virus pathogenicity.....	14
10. Therapeutic Interventions for Exotic Viral Infections.....	15
11. Laboratory Diagnosis of Clinical Illness.....	15
12. Laboratory Instruction	16
C. CONCLUSIONS.....	16
D. BIBLIOGRAPHY	17
E. LIST OF PERSONNEL	17

A. INTRODUCTION

The following is a summary of activities performed under the auspices of this grant, with the Yale University personnel (paid and unpaid) involved in this work listed next to each heading.

The requested funds, previously supplemented by NIH and WHO monies, supported research of the World Reference Center for Arboviruses which: (a) identifies and characterizes approximately 80 suspected arboviruses per year, submitted from U.S. and overseas laboratories, (b) develops new techniques for rapid diagnosis and for characterization of arboviruses and arenaviruses, (c) prepares and distributes reference immune reagents and specific PCR protocols and primers, (d) prepares virus stocks for distribution through WHO regional reference centers and the American Type Culture Collection, (e) prepares and distributes virus antigens, (f) carries out limited virus serosurveys, and (g) disseminates information through WHO, the American Committee on Arboviruses, and by reports in scientific journals.

Emphasis was placed on specific subprojects, which include: (a) nucleotide sequence analysis of flavivirus and arenavirus RNA for molecular epidemiological study of strains, (b) development of viral inactivating agents, for treatment of clinical samples, to render them non-hazardous, (c) use of the extensive reagent bank, to identify emerging viruses by antigenic and genetic methods, and (d) ordering and cataloguing the virus collection into a computer-searchable database.

B. ACTIVITIES

1. Reagents shipped

The following is a list of recipients of viruses, antigens, and/or antibodies requested from the Reference Center collection during 1995-1996.

Virus shipment costs rose disproportionately because of new AITA requirements for special shipping containers (Safe-T-Pak), only one carrier licensed to transport etiologic agents (Federal Express), additional fees and paperwork, and prior certification or acknowledgement of the availability of laboratory facilities meeting biohazard containment levels (e.g., CDC and/or USDA permits).

Table 1. Reagents Distributed 1995-1997

DATE	PRINCIPAL INVESTIGATOR	LOCATION	MATERIAL REQUESTED AND SHIPPED
8/14/95	G. Merano	Mosq Control, FLA	EEERNA
8/16/95	L. Walter	UAF	Diagnostic Supplies
9/21/95	LeDuc, WHO	to Cairo	CCHF, DEN, RVF, WN, YF, ELISA reagents
1/16/96	S. Oberrate	USAMRIID	VEE virus
1/23/96	R. Shope	UTMB	Dengue virus
2/13/96	S. Oberrate	USAMRIID	VEE virus
2/18/96	R. Shope	UTMB	Dengue virus
3/12/96	L. Walters	UAF	ELISA reagents
3/12/96	P.G. Jupp	Ntl. Institute. Vir., South Africa	DEN, YF virus
3/19/96	S. Weaver	UTMB	VEE virus
3/26/96	C. Ramos	Inst Public Health, Mexico	Dengue reagents
4/8/96	R. Shope	UTMB	Dengue virus
4/15/96	B. Hjelle	U of N.M.	primers, TAC ab
4/18/96	C. Ramos	Inst Public Health, Mexico	Documents
4/22/96	Candia	Inst. Pasteur, Paris	RNA
5/6/96	R. Shope	UTMB	SUD K virus
5/13/96	J. Smith	USAMRIID	?RRH
5/22/96	L. Walters	UAF	Reagents
5/30/96	J. Roehrig	CDCFT Collins	VEE virus
6/18/96	R. Shope	UTMB	R 1032 virus
6/24/96	Candia	Institute. Pasteur, Paris	FA slides
6/27/96	A. Da Silva	Sri Lanka	Diagnostic supplies
7/15/96	S. Zaki	CDC	Diagnostic specimens
7/16/96	R. Shope	UTMB (for WHO)	Dengue reagents (2 boxes)
7/17/96	L. Walters	UAF	Documents
7/31/96	B. Innis	WRAIR	TBE, RSSE, YF reagents
8/5/96	R. Shope	UTMB	Chicken sera, Human IgM
8/9/96	J. Patterson	SWF for Bio Res	?RRH
8/20/96	A. Rothman	UMASS	Dengue virus
8/21/96	S. Zaki	CDC.GA	Diagnostic specimens
8/26/96	S. Weaver	UTMB	RNA
8/28/96	N. Kanesa-Thasan	WRAIR	JE, SLE reagents
9.10/96	M. Leyva-Vazquez	UConn for Garcia	Den ELISA reagents
9/11/96	N. Monteny	Laboratory Public Health, Mexico	Mabs for Dengue
9/18/96	R. Novak	Il. Nat. Hist. Surv.	VEERNA
9/30/96	R. Rico Hesse	SWF Bio Res	Dengue virus
10/14/96	L. Walters	UAF	Diagnostic reagents
10/17/96	R. Rico Hesse	SWF Bio Res	Documents
10/21/96	B. Wolf	NJ State Dept. Public Health	EEE mosq. pools
10/22/96	F. Lopoez	Fthill Res. Cent., CA	Diagnostics specimen
11/4/96	O'Neill/Mason	Plum Island	SFV antibodies

2. DNA primers

Synthetic DNA primers were prepared, according to publications of ours and others, for PCR protocols to detect, serotype, and genotype dengue viruses from patient serum samples. These primers were shipped to 6 laboratories (in Mexico, Venezuela, India, and Sri Lanka) for routine diagnostic procedures in national and regional reference laboratories.

3. Virus identification

This activity comprised most of the work done under the auspices of this grant. This work became especially important in arbovirus surveillance in the northeastern part of the United States, during the period June-Nov., 1996. Because of our unique expertise and location, the Yale Arbovirus Research Unit was responsible for the detection and identification of encephalitis viruses in mosquitoes collected in Rhode Island and Connecticut. Eastern equine encephalitis (EKE) virus was isolated from 93 of 1800 mosquito samples collected at various sites within these two states (virus was also detected in Massachusetts and New York). Both State Health Departments relied on our test results and rapid turnaround for the formulation of control policies to protect the public's health. Control methods included mosquito abatement with insecticides of various types, closing of recreational parks, and public awareness campaigns, to reduce exposure. Fortunately, probably because of this rapid response, no human cases of encephalitis occurred during this period. There were deaths in pheasants and emus that were confirmed to be due to EKE during this period, but none in horses.

During the course of this reporting period, some (5) human clinical samples were received from Yale-New Haven and Morristown Hospitals, for arbovirus testing. Most of these cases were diagnosed as probable viral encephalitides, but all were negative in a standard battery of tests. Others were received from suspected dengue cases; these were negative also. Other tests involved detecting specific antibodies (by ELISA and PRNT) to several arboviruses in samples from Alaska, as part of an ongoing, long-term project to detect arbovirus transmission in Alaska natives.

This activity involves virus and antibody maintenance in freezers, having collection information available in electronic form, and responding to reagent requests via electronic mail. In addition to maintaining one of the largest collections of arboviruses in the world YARU also adds to the collection as viruses emerge; this allows us to have a full spectrum of diagnostic reagents available, for the immediate identification of new viruses. During this report period, new low temperature freezers were purchased, to replace nonfunctional units and to avoid failures of freezers which had been in use for over 20 years. Consolidation of samples in larger freezers also allowed us to meet current biosafety guidelines, so that we could store all viruses under lock, in one room.

5. Completing The Yale Catalogue

a. General description:

The YARU virus collection files were transferred to a computers, with new computer searchable software (MS Excel) accessible from both the IBM and Macintosh platforms. We have can now transfered all virus information to the new catalogue which has been stored on Zip disks for circulation to those who want the collection. The Reference Center laboratory maintained its own electronic mail connection, to receive requests and to notify Yale Biosafety offices and others of outgoing shipments of viruses.

The catalogue consists of computer records for nearly 7000 individual specimens containing the following information.

Table 2. Data entries in the Yale catalogue

Names	Taxonomic status	Serogroup	Strain designation	Country of origin and/or country of isolation	Diluent	Volume of sample
Preparation date	Prepared by	Freezer number	Location: Rack/box number	Number of ampoules	Storage form (wet frozen or lyophilized)	Antibody titer information.

The Yale collection consists of viruses, immune reagents, antigens, and serum collections. In past years, each investigator worked independently with unique protocols for accessing reagents. There was no unifying method of identifying reagents, their locations or their preparation methods. Each investigator set his own rules according to specific needs and convenience. We have drawn together these valuable reagents and their documentation into one single collection.

There is now a systematic method for ordering reagents in this collection, for noting duplications and for using backup systems for storing and preserving specimens in case of power failure. We have brought together widely divergent collections, prepared in various manners, into a catalogue which makes these very valuable reagents useful to the greater scientific community.

We have inventoried each of the individual collections. Data has been entered for each item into the computer database. In some instances, this required accessing hard copies of valuable data before computer entry can be effected.

(b) Physical location of the collection

The collection is stored in 20 different freezers and 2 cold rooms. Ten are -20°C freezers and 8 are -80°C freezers. In general, reagents are stored as follows: infectious viruses are stored at -80°C; lyophilized viruses are stored at -20°C ; lyophilized antigens are stored at -4 or at -20 degrees. Storage locations are as follows: 603, 607, 609, 6th floor cold room (4°C), 7th floor cold room (4°C) and a 5th floor walk-in freezer (-20°C). Twelve new freezers have been purchased. Seven old freezers failed mechanically. Seven new freezers replaced the seven broken ones. The walk-in freezer (-20°C) also failed. Materials were moved to 4 new freezers. One old freezer is being held for emergencies.

Table 3. Detailed inventory of storage freezers (-20°C)

Room 603, FS3	Serum collection. Bulk mouse ascitic fluid. Data on cards
Room 603, FS4	Lyophilized virus, entered into the database
Room 603, FS5	Curated virus, lyophilized
Room 603 F8	Curated virus, lyophilized
Room 603, FS7	Various serum collections,
Room 603, FS9	Various serum collections,
Room 603, FS10	Various serum collections,
Room 603, FS13	Virus reagents
Room 603, F6	Curated lyophilized virus, serum and other reagents
Room 607, YM2	Serum collections, and miscellaneous reagents
Room 612, STP2	NIH antigens, lyophilized viruses,
Room 612, STP3	A variety of reagents,

6. Local Field Surveillance , 1995-1996

a. Rhode Island Arbovirus Surveillance Program: Summary

Table 4. Descriptive data: summary of mosquito surveillance program

Start date	End date	Number of shipments*	Number of mosquito pools	Number of mosquitoes
June 20, 1996	October 28, 1996	21	1,125	39,024

*received from the Rhode Island Office of Mosquito Abatement

Table 5. Results of ri state mosquito-virus isolation studies

Viruses identified	Date of first isolation	Number of isolations	Date of last isolation	Other species tested
Jamestown Canyon	July 11, 1996	1	NA	NONE
Highlands J	August 8, 1996	48	October 21, 1996	NONE
EEE	August 8, 1996	57	October 28, 1996	2/4 emu 0/1 pheasant

(brain, blood, or both)

b. Connecticut Arbovirus Surveillance Program: Summary

Table 6. Summary of CT state mosquito surveillance program

Start date	End date	Number of shipments*	Number of mosquito pools	Number of mosquitoes
September 9, 1996	October 25, 1996	25	639	7,281

*received from the Connecticut Agriculture Experiment Station

Table 6. Results Of CT State Mosquito-Virus Isolation Studies

Viruses identified	Date of first isolation	Number of isolations	Date of last isolation	Other species tested
Highlands J	September 6, 1996	21	October 11, 1996	NONE
EEE	September 9, 1996	36	September 28, 1996	NONE

c. Summary Of New England Surveillance Program

Comprehensive surveillance for virus infected mosquitoes has been done at Yale for the State of Rhode Island. In 1996, there were more isolations of EEE from mosquitoes than in any previous year in recent history. These isolations attracted considerable attention from the local and national media because of fear that children would be infected with EEE, a very serious disease in children. Mosquito abatement programs were immediately instituted in Rhode Island. Concern and fear developed in neighboring regions of the State of Connecticut.

The study of EEE has also been a collaborative effort between investigators at Yale and investigators at the Connecticut Agriculture Station in Hamden, CT in previous years. As in Rhode Island, mosquito abatement programs were instituted immediately in areas suggested by the results of the mosquito isolations at Yale.

7. International Field Surveillance for Arboviruses

a. Turkey - Mark L. Wilson

As part of related studies on arthropod-borne disease agents in an area of southeastern Turkey undergoing extensive environmental change from a major irrigation project there, we collected mosquitoes in hopes of isolating arboviruses. This area of the Near East, which includes northern Syria, western Iraq, and Iran has not been surveyed recently due to political and logistic problems. We anticipated that the region of southeastern Turkey, bordering these three countries, presented an opportunity to attempt arbovirus isolations from the natural vector fauna of the region.

During June - August 1996, Pia MacDonald (Yale MPH student) worked with Turkish medical entomologists (Dr. H. and M. Kasap) in an attempt to capture and preserve mosquitoes for isolation efforts. CDC light traps were set in various sites during the hot/dry season to capture adult insects. Unfortunately, only a few mosquitoes were captured (mostly *Anopheles* species), even though traps were set in habitats likely to be suitable for adults (near streams, inside dwellings, near reservoirs, etc.). In addition, it was not feasible to preserve these few specimens captured in liquid nitrogen. Nevertheless, the identity of these few *Anopheles* species was determined, and data on the incidence of malaria by provinces in the region was calculated. This work was part of Ms. MacDonald's MPH thesis.

Additional studies of sandfly vectors of cutaneous leishmaniasis were undertaken, in part through the use of CDC traps purchased from the grant. These observations were designed to evaluate how habitat characteristics derived from satellite image data, and related to demographic and environmental variables, might influence transmission. Results of these studies on malaria and cutaneous leishmaniasis were presented at the 1996 annual meeting of the American Society of Tropical Medicine and Hygiene, and have been submitted for publication or are in preparation.

b. Sri Lanka - Aravinda de Silva

This project involved collecting serum from toque macaque (*Macaca sinica*) monkeys in Polonnaruwa, Sri Lanka, to attempt to identify a dengue-like flaviviruses that had presumably infected them (Peiris *et al.* , *J. Med. Primatol.* 22: 240-245, 1993). Previous studies had shown that, of 68 animals sampled in 1987, 35% had dengue type 2 antibody (PRNT) titers of over 1:160. No virus isolations had been made and the infecting virus could not be identified.

We relied on newer serologic (IgG ELISA) and genetic (PCR) techniques to try to determine the identity of the infecting flavivirus. During July-Oct. 1995, 246 serum samples were collected by our collaborator, Dr. Wolfgang Dittus, of the National zoological Park, Smithsonian Institution, Washington, DC, from toque macaques ranging in age from 4 most to 33 yrs., from the same population group as that reported earlier. The ELISA test was done using various antigens from flaviviruses which had been reported to circulate in Sri Lanka and India (dengue, Japanese encephalitis, yellow fever, West Nile, Zika); only dengue-reactive antibodies were detected. The results showed that only animals over the age of 9 years had antibodies to a dengue-like virus and the study of several animals in the 8-9 year range allowed us to pinpoint the time of infection during Aug. 1986-Jan. 1987. Because none of the younger macaques had antibodies, this virus seemed to have disappeared from this ecological niche during the period 1987-1995. PCR tests on several of the positive sera were negative and we realized that the samples had previously been heat-inactivated, probably destroying any available viral RNA template. We are presently trying to obtain serum samples from 1986 or earlier, which have been stored at -20 C or below, without heat inactivation, so that we may attempt to enzymatically amplify the infecting virus and definitely determine its identity. One trip was made to the National Zoo, in Washington, to go through the serum collection held there, to find the appropriate samples for PCR, but this was unsuccessful. We are still checking for aliquots in collections stored at Columbia University and at a private company in Texas.

The results of this study could potentially provide very important information concerning the origin of epidemic strains of dengue virus and whether these viruses are exchanged between human and non-human primate populations in an island such as Sri Lanka. There have been numerous human epidemics there since the 1970's, although most have been limited to the western, urban areas of the country (Colombo). Because the macaque population under surveillance is limited to the north-central area of the country, we hypothesized that this possible dengue virus could represent another example of a sylvatic strain of dengue which does not seem to infect humans.

c. Venezuela - Katrin Leitmeyer

During the period June 9-18, 1996, a trip was made to Venezuela, to collect samples of Guanarito virus from humans and rodents. This project involved obtaining epidemiologic and clinical information on human hemorrhagic fever cases and ecologic information on infected rodents (*Zygodontomys brevicauda*), to determine the geographic and temporal distribution of this virus in Venezuela. This work was done in collaboration with the National Institute of Hygiene, in Caracas. Another objective was to obtain unpassaged or low passage virus samples, for optimization of the PCR test being developed for specific identification of arenaviruses (see below).

The samples obtained during this visit included 9 human serum aliquots from hemorrhagic fever cases hospitalized during the period Feb. 1991 - Mar. 1996, in Portuguesa State. Rodent samples included 26 virus isolates from *Z. brevicauda*, collected in the same area, during the period Feb. 1994 - Feb. 1995. We are currently determining the nucleotide sequence of amplified viral RNA, to understand the population dynamics of this virus in the rodent reservoir (ts) and its human host. This information will eventually provide a better estimate of Guanarito virus mutation frequencies in nature, an important consideration in the design of vaccines.

d. Evaluation Of A Dengue Fever Intervention In Barbados, W.I.

Sonya Greaves-Ponder

Gregory H. Tignor

In 1993, the Ministry of Health (MOH) Barbados joined with the Pan American Health Organization (PAHO), to conduct a knowledge, attitude and practice (KAP) survey that provided information to initiate an *Aedes aegypti* community integrated dengue control program in Barbados.

The outcomes of the 1995 and 1993 KAPs were compared to analyze the efficacy of the intervention that followed the 1993 KAP survey. The results indicate slightly increased general knowledge of dengue fever (95.0% vs. 91.3 %, $p < 0.05$) with increased specific knowledge of the signs and symptoms of dengue fever (36.1% vs. 14.3%, $p < 0.05$). There is also increased knowledge of the mechanism of disease transmission (73.2% vs. 64.9%, $p < 0.05$) and of mosquito breeding sites (81.7% vs. 28.0%, $p < 0.05$). However, the results also show that the study population (91.9%) remained ill-informed of the biting habits of the dengue mosquito vector, *Aedes aegypti* mosquito. A large majority of the study population (68%) thought the vector mosquito bit at night. The study population protect themselves at night, but they do not protect themselves during the day when the *Aedes aegypti* mosquito bites.

8. Development of Rapid Diagnostic Reagents

a. Hemorrhagic Fever PCR - Katrin Leitmeyer

The goal of this project was to develop a broadly reactive and non-hazardous PCR protocol for detection of hemorrhagic fever viruses (arenaviruses). Patient and rodent serum was first treated with a monophasic solution of phenol and guanidine isothiocyanate; this step inactivates any infectious agents while conserving viral nucleic acids for subsequent enzymatic amplification (shift from BL4 to BL2 containment levels). The viral RNA was extracted and reverse transcribed using a commercial kit and a specially-designed DNA primer tARE/3'end: CGCACAGTGGATCCTAGGC, 19-mer). The resulting cDNA template was used in a PCR, with the addition of a virus-sense primer (ARE/2595V: CTRARRTTTANCCWGADATRT, 22-mer), to generate a 800 bp fragment, after 40 thermal cycles. Different PCR conditions were tested (primers, pH, temperature, salt concentration, etc.) to determine optimal yields. These products were sequenced manually with commercially available kits.

The resulting nucleotide sequences were used to distinguish two arenaviruses (Guanarito and Pirital) which are transmitted simultaneously in *Sigmodon alstoni* rodents in

Venezuela. This rapid diagnostic test was used to investigate the temporal and geographic range, host species, and human pathogenicity of these and other arenaviruses in this country. This test was also used to distinguish the etiology of human cases of hemorrhagic fever, specifically to exclude other viral agents such as dengue, yellow fever, and hantaviruses.

b. Virus Stability Study: Effects Of Different Virus Inactivating Methods On Arbovirus Antigens Monath, T., Rico Hesse, R., Tirrell-Peck

In collaboration with Dr. Thomas Monath, of Pentose Pharmaceuticals Inc., we began testing the virus inactivation potential of several polymers of ethylenimine (i.e., pentose). We chose Venezuelan equine encephalitis (VEE) viruses as models for testing inactivation and structural protein (antigenic) stability because of our ample experience and the availability of numerous monoclonal antibodies with very sensitive reactivities with these viruses. Several strains of VEE viruses were successfully inactivated by a pentose trimer, when placed in normal human serum, while retaining all detectable antigenic sites. These studies are continuing, so that we may optimize treatment conditions to suit application of these inactivating agents in a clinical setting. The potential use of these agents includes: treatment of blood or other patient fluid samples, to render them non-hazardous for further examination of clinical parameters and virus detection; use of these agents for vaccine production, especially for emerging viruses, for rapid turnaround of killed virus immunogens; elimination of adventitious viruses in many commercial or industrial preparations.

9. Advances in Molecular Epidemiology

a. Arenavirus phylogeny - Jean-Paul Gonzalez

Most of the activity for this project was limited to the study of Sabia virus, due to limitations on the growth of these agents in our facilities, because of biological safety concerns. We had already prepared numerous aliquots of extracted viral RNA of several arenaviruses, but our attempts to amplify some of these by PCR were unsuccessful.

Sabia virus, one of five arenaviruses from South America known to cause hemorrhagic fever in humans, emerged in 1990 when it was isolated from a fatal case in Sao Paulo, Brazil. Subsequently, it has caused two laboratory-acquired infections. Its natural distribution and host are still unknown. Using viral RNA and multiple polymerase chain reaction products as templates, the nucleotide sequence of the small (S) RNA segment of Sabia virus, which codes for the nucleocapsid and glycoprotein precursor, was determined. This virus shares an ambisense genome in common with other arenaviruses, although it has a unique predicted three stem-loop structure in the S RNA intergenic region. Phylogenetic analysis of a portion of the N gene sequence confirmed that this virus is distinct from all other members of the arenavirus family and that it shares a progenitor with Junin, Machupo, Tacaribe, and Guanarito viruses. We were also able to establish that the analysis of only 730 nucleotides was required for obtaining reliable phylogenies and this approach could potentially be used to identify and classify subsequent emerging arenaviruses. This approach was adapted to a rapid PCR protocol which could distinguish arenaviruses from several countries in the Americas.

b. Dengue virus pathogenicity - Rebeca Rico-Hesse

Ongoing studies on the molecular evolution of dengue type 2 viruses provided new information on the origin of more pathogenic viruses belonging to this serotype. Other research projects done in Thailand (sponsored by NIH) have also contributed to a better understanding of severe dengue pathogenesis. Our working hypothesis is that both host (immune status) and viral (genetic) factors determine the potential for dengue fever progressing to hemorrhagic fever in the infected individual.

The recent emergence and spread of dengue hemorrhagic fever in the Americas has been a major source of concern. Efforts to control this disease are dependent on understanding the pathogenicity of dengue viruses and their transmission dynamics. Pathogenicity studies have been hampered by the lack of in vitro or in vivo models of severe dengue disease. Alternatively, molecular epidemiologic studies which associate certain dengue virus genetic types with severe dengue outbreaks may point to strains with increased pathogenicity. The comparison of nucleotide sequences (240 bp) from the E/NS1 gene region of the dengue virus genome has been shown to reflect evolutionary relationships and geographic origins of dengue virus strains (*Virology* 174:479-493, 1990). This approach was used to demonstrate an association between the introduction of two distinct genotypes of dengue type 2 virus and the appearance of dengue hemorrhagic fever in the Americas. Phylogenetic analyses suggest that these genotypes originated in Southeast Asia and they displaced the native, American genotype in at least 4 countries. Vaccination and other control efforts should therefore be directed at decreasing the transmission of these "virulentH" genotypes.

10. Therapeutic Interventions for Exotic Viral Infections

There was no activity in this area; YARU clinical personnel did not have to respond to emergencies of this type. Drs. Michelle Barry and Frank Bia therefore did not collect salaries for this activity.

11. Laboratory Diagnosis of Clinical Illness

Historically, this unit has provided services to CT and surrounding communities for diagnosis of CNS disease of uncertain origin. This service has extended beyond the arbovirus viruses to includes other viruses such as herpes, rabies, CMV, and LCMV. It is a service that is highly valued by hospitals in the surrounding community. The number of cases on which this unit collaborates varies from year-to-year, depending on the number of CNS infections observed in the community. We cannot predict how many times we will be called upon to provide assistance, but we stand ready. We keep a supply of rapid diagnostic reagents and materials on stock to enable us to provide rapid and certain answers to diagnostic questions involving arboviruses.

12. Laboratory Instruction

Research Associates involved in the Reference Center activities attended a special course on how to meet new virus shipping requirements (AITA, CDC/NIH, FedEx, USDA, Yale Biosafety) so that we could respond promptly to requests from others. In addition, eight Yale University students and other investigators (from Mexico, Venezuela, etc.) were trained in basic protocols used for virus identification (e.g., PCR, ELISA, HI).

The Down's Fellowship attracts a great many applicants. Many applicants need training in laboratory techniques in order to conduct their research projects in foreign countries. They use the facilities of the arbovirus unit to learn the requisite techniques for use in their field studies. This is appropriate because the fellowship is named for Wilbur Downs, a former Director of the Arbovirus Unit. We expect to continue to play this support role for the School of Public Health during 1996-1997.

In addition, postdoctoral and other fellows associated with the Vector Biology Unit often come to the arbovirus laboratory to learn specific techniques in arbovirology.

C. CONCLUSIONS

In addition to maintaining one of the largest collection of reference viruses in the world, the Reference Center served functions critical for maintaining the public's health and provided important information concerning the origin, transmission and control of arboviruses. We provided training and reagents to both national and foreign laboratories, so that they might also contribute to the detection and control of these viruses. The results reported here and those obtained by others are important in understanding the epidemiology of viruses exotic to the United States, including those considered to be emerging in other areas of the world and therefore potentially hazardous to military and civilian personnel of this country.

The final months of this contract were spent in efforts to move this collection from Yale to CDC where the activities that we have conducted can be continued.

D. BIBLIOGRAPHY

1. Publications

Gonzalez, J.P.J., Bowen, M.D., Nichol, S.T. and R. Rico-Hesse (1996~). Genetic characterization and phylogeny of Sabia virus, an emergent pathogen in Brazil. *Virology* 221:318-324.

Wang, E., Weaver, S C., Shope, R.E., Tesh, R.B., Watts, D.M., and A.D.T. Barrett (1996). Genetic variation in yellow fever virus: duplication in the 3' noncoding region of strains from Africa. *Virology* 225:274-281.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J.R., de Mesa, M.T., Nogueira, R.M.R., and Travassos da Rosa, A. (1997). Origins of dengue serotype 2 viruses associated with increased pathogenicity in the Americas. *Virology*. In press.

Alptekin D, Kasap M, Kasap H, Aksay S, Wilson ML. Sandflies (Diptera: Psychodidae) associated with an epidemic of cutaneous leishmaniasis in Sanliurfa, Turkey. *J. Med. Entomol.* (submitted).

2. Meeting Abstracts

Rebeca Rico-Hesse

Am. Soc. Trop. Med. Hyg., Dec. 1996: "Evolution of dengue type 2 viruses associated with increased pathogenicity in the Americas".

International Symposium on Factors in the Emergence of Arbovirus Diseases, Annecy, France, Dec. 1996: "Molecular evolution of dengue type 2 virusesU.

Mark L. Wilson

Am. Soc. Trop. Med. Hyg., 1996: "*Phlebotomus* sandflies associated with epidemic cutaneous Leishmaniasis in Sanliurfa, Turkey"; "Epidemic reemergence of cutaneous Leishmaniasis in Saliurfa, Turkey: Epidemiological characterization and hypotheses".

E. LIST OF PERSONNEL

Gregory H. Tignor, Associate Professor

Rebeca Rico-Hesse, Associate Professor
(receiving salary)

Mark L. Wilson, Associate Professor

Katrin Leitmeyer, Postdoctoral Associate

Shirley Tirrell-Peck, Senior Research Associate

Joann Zamparo, Research Associate

Sherry Calcasaco, Secretarial Assistant

Mary Ganues, Laboratory Aide

E. Kruglov (filling in for M. Ganues)